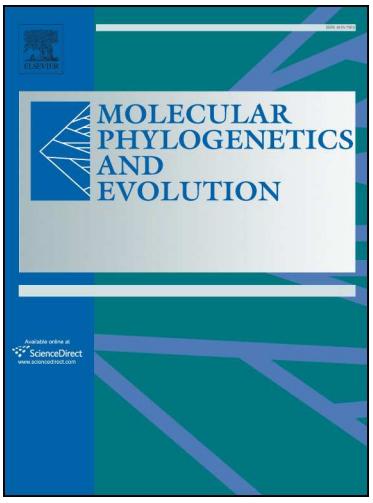

The Near East as a cradle of biodiversity: a phylogeography of banded newts (genus *Ommatotriton*) reveals extensive inter- and intraspecific genetic differentiation

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Article title: **The Near East as a cradle of biodiversity: a phylogeography of banded newts (genus Ommatotriton) reveals extensive inter- and intraspecific genetic differentiation**

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ABSTRACT

The banded newt (genus Ommatotriton) is widely distributed in the Near East (Anatolia, Caucasus and the Levant) – an understudied region from the perspective of phylogeography. The genus is polytypic, but the number of species included and the phylogenetic relationships between them are not settled. We sequenced two mitochondrial and two nuclear DNA markers throughout the range of Ommatotriton. For mtDNA we constructed phylogenetic trees, estimated divergence times using fossil calibration, and investigated changes in effective population size with Bayesian skyline plots and mismatch analyses. For nuDNA we constructed phylogenetic trees and haplotype networks. Species trees were constructed for all markers and nuDNA only. Species distribution models were projected on current and Last Glacial Maximum climate layers. We confirm the presence of three Ommatotriton species: O. nesterovi, O. ophryticus and O. vittatus. These species are genetically distinct and their most recent common ancestor was dated at ~25 Ma (Oligocene). No evidence of recent gene flow between species was found. The species show deep intraspecific genetic divergence, represented by geographically structured clades, with crown nodes of species dated ~8-13 Ma (Miocene to Early Quaternary); evidence of long-term in situ evolution and survival in multiple glacial refugia. While a species tree based on nuDNA suggested a sister species relationship between O. vittatus and O. ophryticus, when mtDNA was included, phylogenetic relationships were unresolved, and we refrain from accepting a particular phylogenetic hypothesis at this stage. While species distribution models suggest reduced and fragmented ranges during the Last Glacial Maximum, we found no evidence for strong population bottlenecks. We discuss our results in in the light of other phylogeographic studies from the Near East. Our study underlines the important role of the Near East in generating and sustaining biodiversity.

Keywords: Anatolia; historical biogeography; Levant, molecular dating; phylogeny; species distribution modelling

INTRODUCTION

Phylogeography reconstructs phylogenetic patterns in space and time to identify the drivers that shape biodiversity within taxa (Avise, 2000; Hickerson et al., 2010). The Mediterranean Basin is considered a near-ideal laboratory for phylogeographical studies (Hewitt, 2011). The collision of the Eurasian, African and Arabian tectonic plates, from the Late Eocene (37-34 Ma) onwards, caused major geological changes in the Mediterranean Basin, as marine barriers were re-arranged and mountains formed (Meulenkamp & Sissingh, 2003; Popov et al. 2006). This process left its signature in the phylogeography of the species inhabiting the region (Hewitt, 2011). While the Quaternary (2.6 Ma - present) glacial cycles resulted in drastic climate shifts in temperate Eurasia, the climate in the Mediterranean Basin remained relatively stable (Stewart et al., 2009; Hewitt, 2011). As a consequence, many species had a continuous presence within the Mediterranean Basin, while they only periodically colonized more northern areas during interglacials – and subsequently were wiped out during the next glacial cycle (Hewitt, 2000).

Because of the interaction of geographic instability and climatic stability, many Mediterranean species are characterized by deep intraspecific lineages (Oosterbroek & Arntzen, 1992; Bilgin, 2011). The Mediterranean Basin features a high concentration of biodiversity and is considered a biodiversity hotspot (Myers et al., 2000). Complex phylogeographic patterns are particularly well documented for the Iberian, Italian and Balkan peninsulas (Hewitt, 2011). Despite the similar geologic and climatologic background of the Near East, historical biogeographical studies of this region are underrepresented in the phylogeographical literature (Beheregaray, 2008; Riddle, 2016).

Most amphibian species, especially salamanders, are particularly influenced by geological disruptions and climate oscillations, due to their limited dispersal capabilities and strong dependence on water, often resulting in a pronounced phylogenetic structure (Vences & Wake, 2007). Seven Salamandridae genera (Lissotriton, Lyciasalamandra, Mertensiella, Neurergus, Ommatotriton, Salamandra, and Triturus) occur in the Near East. Phylogeographical studies of these genera revealed long term in situ evolution and survival during glacial cycles (Steinfartz et al., 2000; Tarkhnishvili et al., 2000; Veith et al., 2008; Wielstra et al., 2010; Hendrix et al., 2014; Pabijan et al., 2015). However, banded newts (genus Ommatotriton) remain to be studied in-depth. Because Ommatotriton is the most wide-spread salamander genus within the Near East (Sparreboom, 2014), a phylogeographic survey is expected to be insightful regarding the region's historical biogeography.

Ommatotriton is endemic to the Near East (Fig. 1). The genus was considered a single species, O. vittatus (Gray, 1835), with a disjunct northern and southern distribution, until Litvinchuk et al. in 2005 proposed that the northern banded newt range segment should be regarded as a separate, distinct species, O. ophryticus (Berthold, 1846). This treatment is now generally accepted (e.g. Sparreboom, 2014; Frost, 2016). A recent proposal by Bülbül & Kutrup (2013) to split the northern banded newt range segment into two species, O. ophryticus in the east and O. nesterovi (Litvinchuk, Zuiderwijk, Borkin and Rosanov, 2005) in the west, is not universally supported. For convenience we refer to the three taxa as species from here on. The phylogeny of Ommatotriton is unclear. Considering three Ommatotriton species, three branching orders are possible and each is supported by existing data. Phylogenetic hypothesis I ((O. ophryticus, O. vittatus), O. nesterovi) is supported by mtDNA data (Bülbül & Kutrup 2013), II ((O. ophryticus, O. nesterovi), O. vittatus) by allozyme data (Arntzen & Olgun, 2000; Litvinchuk et al., 2005), and III ((O. vittatus, O. nesterovi), O.

ophryticus) by the number of rib-bearing vertebrae (Arntzen & Olgun, 2000; Arnzen et al., 2015 Litvinchuk et al., 2005).

Previous studies dealing with the inter- and intraspecific phylogenetic patterns of Ommatotriton suffered from limited sampling. We here conduct range-wide sampling. We use mitochondrial and nuclear DNA (mtDNA and nuDNA) markers to determine inter- and intraspecific phylogenetic relationships, conduct fossil-based time calibration and test for interspecific hybridization. Additionally, species distribution models are constructed to evaluate the impact of the Quaternary climate oscillations, and compared to historical demographic reconstructions. We identify interspecific and intraspecific phylogeographical patterns of Ommatotriton and discuss our findings in the context of a comparative phylogeography of the Near East. Our study highlights the importance of the Near East as a biodiversity hotspot.

MATERIAL AND METHODS

2.1 Sampling and sequencing

We collected tissue samples of 1-3 individuals (average 2.6, total 106) from 40 localities (Fig. 1, Table A.1). The Thermo Scientific KingFisher Purification System was used to extract DNA. Fragments of two mitochondrial protein coding markers were sequenced: cytochrome b (cyt b, 786 bp) and cytochrome c oxidase I (COI, 658 bp). We added COI sequences for 13 individuals from five additional localities from Smith et al. (2008). Fragments of two unlinked nuclear protein coding markers (Hendrix et al., 2014; Shen et al., 2012), KIAA1239 (600 bp; hereafter KIAA) and SACS (624 bp), were sequenced for a single individual per locality, but included all three individuals for the three O. ophryticus and three O. nesterovi

locations closest to their parapatric contact zone as defined by the mtDNA data (see Results, total 47, Table A.1).

We adjusted the 'universal' cyt b primers (MVZ-15-NP, MVZ-16-NP) from Moritz et al. (1992) and COI primers (VF1-d, VR1-d) from Ivanova et al. (2006) to match the full mitogenome of O. nesterovi (Zhang et al., 2008; Table A.2). We designed Ommatotriton-specific primers based on a KIAA and a SACS (Shen et al., 2012) sequence for O. nesterovi presented in Hendrix et al. (2014). In cases where no PCR product could be obtained, presumably due to degraded DNA, internal primers were designed to amplify stretches of 100-300 bp (three primer sets for COI, KIAA and SACS, and four for cyt b; Table A.3).

PCR conditions for DNA amplification were: initial denaturation for 180 s at 94°C, 35 cycles with 30 s denaturation at 94°C, 30 s annealing at 58°C and 60 s extension at 72°C, and a 240 s final extension step at 72°C. Sanger sequencing was done commercially on an ABI 3730xl DNA analyser at BaseClear B.V., Leiden, the Netherlands. Sequences were assembled in SEQUENCHER 4.10.1 (Gene Codes Corporation, MI USA) and aligned in MESQUITE 3.04 (Maddison & Maddison, 2015). Nuclear alleles were phased with the PHASE 2.1 algorithm (Stephens & Donnelly, 2003) implemented in DNASP 5 (Librado & Rozas, 2009). GenBank accession numbers are listed in Table A.1.

2.2 Phylogenetic analyses

The mtDNA alignment was divided into six partitions, by marker (COI and cyt b) and by codon position (first, second and third). Lissotriton vulgaris and Neurergus strauchii were added as outgroups (data from Zhang et al. 2008; Table A.2). Maximum likelihood (ML) analysis was performed with RAXML 8.2.4 (Stamatakis, 2014), with 1000 rapid bootstrap replicates and a search for the best scoring maximum likelihood tree. The nucleotide substitution model GTR+G was used for each partition, as recommended in the RAXML

manual. Bayesian phylogenetic reconstruction was performed with MRBAYES 3.2.6 (Ronquist et al., 2012). We determined the best fitting models of sequence evolution for each of the six partitions based on the Bayesian information criterion (Guindon & Gascuel, 2003) in JMODELTEST 2.1.7 (Darriba et al., 2012; Table A.4). A Markov Chain Monte Carlo search was run for a million generations under default parameters (two runs, four chains, with temperature 0.2), a sampling frequency of 0.001 and a burn-in of 25% of generations. State frequencies were unlinked and rates were set to vary across each of six partitions. TRACER 1.6 (Rambaut et al., 2014) was used to confirm stabilisation within and convergence between runs (all ESS values > 200). RAXML and MRBAYES were run on the Cipres Science Gateway (Miller et al., 2010). Trees were also reconstructed for each mtDNA marker separately, following the same procedure as for the concatenated mtDNA data.

Phylogenetic trees for nuDNA were constructed using the same approach as for mtDNA, with Neurergus strauchii as outgroup (data taken from Hendrix et al., 2014). Because JMODELTEST suggested two out of three substitution models to be identical for codon positions within nuclear markers (Table A.4), each marker was analysed unpartitioned to avoid over-parameterisation of the analysis. Because these trees showed little divergence (Fig. B.7-B.10), we additionally constructed minimum spanning haplotype networks (Bandelt et al., 1999) for each nuclear marker in POPART 1.7 (Leigh et al., 2016).

*BEAST, implemented in BEAST 1.8.3 (Heled & Drummond, 2010; Drummond et al., 2012), was used to construct a species tree for the nuDNA markers. Species were set as operational taxonomic units. We selected the best fitting model per marker in JMODELTEST (Table A.4). We performed a species tree analysis including all mtDNA and nuclear DNA markers (two twenty million generation runs), and a species tree analyses using only the two nuclear DNA markers (one ten million generation run) with a sampling frequency of 0.001. TRACER 1.6 was used to confirm stabilisation and convergence as for MRBAYES. A burn-in of

10% was removed when combining log and tree files in LOGCOMBINER (BEAST 1.8.3). TREEANNOTATOR (BEAST 1.8.3) was used to construct a maximum clade credibility tree with mean node height.

2.3 Molecular dating

COI and cyt b sequences for all Salamandridae genera (Table A.2) were taken from Zhang et al. (2008). We included 14 Ommatotriton haplotypes, one randomly chosen haplotype from each phylogeographical groups (see Results, Fig 2a) and a distinct haplotype found at a single locality only (Nes_08 from locality 4). We excluded the cyt b sequence for Ichthyosaura alpestris, because it contained a large number of unique non-synonymous substitutions suggestive of a pseudogene (see also Steinfartz et al., 2007). BEAST 1.8.3 was used to generate a calibrated phylogeny, employing seven fossil-based calibration points within Salamandridae, the family to which Ommatotriton belongs, following the procedure outlined in Wiens et al. (2011; Table A.5). To ensure that the calibration points fitted within the age constraints, we constructed a starting tree adapted from a RAXML tree with the R package 'APE' (Paradis et al., 2004). Four runs of 20 million generations were executed on the Cipres Science Gateway with nucleotide substitution models selected with JMODELTEST (Table A.4) and a sampling frequency of 0.001. Log and tree files were combined and convergence was checked as above.

2.4 Species distribution modelling

We combined our localities of species occurrence with those in Borkin et al. (2003). This yielded 75 localities for O. vittatus, 53 for O. nesterovi and 233 for O. ophryticus. Localities were assigned to species based on ranges suggested by our genetic analyses. We used bioclimatic variables from the WorldClim database 1.4 (Hijmans et al., 2005) as explanatory

variables. Following Guisan & Thuiller (2005) and Peterson (2011), we selected variables with a Pearson's correlation < 0.7 that were considered biologically meaningful: isothermality (bio3), temperature seasonality (bio4), mean temperature of wettest quarter (bio8), mean temperature of driest quarter (bio9), precipitation of wettest quarter (bio16) and precipitation of driest quarter (bio17).

We used MAXENT 3.3.3k (Phillips et al., 2006) to construct species distribution models for each Ommatotriton species. Because the area on which species distribution models are calibrated influences model parameters considerably (Stokland et al., 2011), we restricted sampling of pseudo-absence data to a 200 km buffer zone around Ommatotriton localities (VanDerWal et al., 2009). Feature type was restricted to hinge features for a smoother model fit, emphasising trends rather than idiosyncrasies of the data (Elith et al., 2010). We confirmed that the models performed better than random by testing them for significance against a null model derived from random localities, following Raes & ter Steege (2007). Random point data (99 replicates per analysis) created with ENMTOOLS 1.3 (Warren et al., 2010). Models were projected on climate reconstructions for the Last Glacial Maximum (~21 ka), based on the MIROC (Sueyoshi et al., 2013) and CCSM (Brady et al., 2013) climate simulations, available from WorldClim.

2.5 Population dynamics

Bayesian skyline plots (Ho & Shapiro, 2011) were generated with BEAST for 40 O. nesterovi, 40 O. ophryticus, and 39 O. vittatus mtDNA sequences with 6 million generations. Parameters were kept at default except for the nucleotide substitution models (selected with JMODELTEST; Table A.4) and the estimated total substitution rate (4.802×10^{-3} substitutions My^{-1} from the molecular dating analysis). Mismatch analyses were performed per species in DNASP (Table A.6). We used COI data only because DNASP is unable to handle missing data,

and several cyt b sequences were not available (Table A.1). The observed haplotype frequencies were compared to haplotype frequencies expected at constant population size. The statistics R_2 and *Fu's Fs* (Ramos-Onsins & Rozas, 2002) were calculated to test for significance ($R_2 < 0.05$ and *Fu's Fs* < 0.02), using coalescent simulations with 1000 generations.

RESULTS

For COI the number of segregating sites (S) is 214, nucleotide diversity (π) is 0.119, and total number of mutations (θ) is 71.26, for cyt b 100, 0.114, and 30.55, for KIAA 20, 0.007 and 4.11 and for SACS 17, 0.006 and 3.34. The mtDNA phylogenetic tree revealed three monophyletic groups, corresponding to the three Ommatotriton species (summarized in Fig. 2a, detailed MrBayes and RAxML trees in Fig. B.1 and B.2). The most recent common ancestor of Ommatotriton was dated at 24.5 Ma [95% HPD confidence interval (CI) 14.6-35.8 Ma, Fig. 2b]. Ommatotriton nesterovi, O. ophryticus and O. vittatus crown nodes were estimated at 13.0 Ma (CI 4.9-22.3 Ma), 8.6 Ma (CI 2.1-17.2 Ma) and 12.5 Ma (CI 5.5-20.8 Ma). The phylogenetic tree based on both mtDNA markers suggested O. nesterovi and O. ophryticus as sister species, but with low support, independent of the method used (Fig. 2a). Phylogenetic trees for the individual mtDNA markers strongly supported the three monophyletic clades, while relationship among clades were poorly supported (Fig. B.3-B.6). The species tree based on nuDNA only suggested that O. ophryticus and O. vittatus are sister species, with high support (posterior probability of 0.98; Fig. 3c), while adding mtDNA to the *BEAST analysis resulted in a lower support for this clade (0.75).

For nuDNA phylogenetic trees (BI and ML, Fig. B.7-B.10), weak support was found for a monophyletic O. nesterovi. Ommatotriton vittatus was suggested, albeit with low

support, to be either monophyletic or paraphyletic and nested within O. ophryticus, reflecting the relationships found in the haplotype network. In the KIAA network haplotypes for each species are clustered, while in the SACS haplotype network O. nesterovi was split up in two groups (Fig. 3a, 3b). We found no evidence for nuDNA haplotype sharing between species as defined by mtDNA (Fig. 2a, 3a, 3b).

Within species, 13 distinct monophyletic mtDNA clades representing geographically coherent groups were found (Fig. 1 and Fig. 2a). The sole exception was haplotype Nes_08 from locality 4, which is placed in a sister position to phylogeographic groups A and B and occurs in the geographic range of group A. Intraspecific phylogeographical groups were found to be more closely related to geographically more closely located phylogeographic groups than to groups located further apart, with the exception of the I+M-clade from Israel and the Adana Basin. For nuDNA, intraspecific differentiation was low (Fig. 3 and Fig. B.7-B.10). For the SACS gene, individuals from group I (localities 30 and 31) were relatively diverged from other individuals of O. vittatus (groups J-M). The KIAA marker suggested divergence of western (group E; localities 15-17) and eastern (groups F-H; localities 19-29) O. ophryticus, in line with the mtDNA-based phylogenetic tree. The mtDNA phylogeographic groups, however, were not recovered in the nuDNA analyses.

Projections on Last Glacial Maximum climate reconstructions showed the potential distribution for the three species was reduced and fragmented compared to the present (Fig. 4 and Fig. B.11). For O. vittatus, the range reduction was less pronounced than for O. nesterovi and O. ophryticus. The potential distributions of O. nesterovi and O. ophryticus overlap considerably at the present day and much less so at the Last Glacial Maximum. Unsuitable environmental conditions separated O. vittatus from the other two species during either period. Bayesian skyline plots based on mtDNA indicated a more pronounced dip in effective population size for O. nesterovi at ~0.5 Ma than for O. ophryticus and O. vittatus at ~0.05 Ma

and at ~0.5 Ma (Fig. B12). Mismatch analyses indicated that allele frequencies did not differ from the allele frequency expected for a stable population over time (Fig. B.11, Table A.6).

DISCUSSION

4.1 Biogeographical scenario for three genetically distinct species

The mtDNA phylogeny for Ommatotriton reveals three genetically distinct clades, which is in line with the proposal to recognize three banded newt species: O. nesterovi, O. ophryticus and O. vittatus. While reciprocal monophyly is not significantly supported for individual nuDNA markers, crucially none of the nuclear haplotypes are shared between species, not even in the region where the ranges of O. nesterovi and O. ophryticus approach one another, and where we sampled more densely (Fig. 1).On the basis of these results we accept the three species treatment.

Hybridization is not a likely cause of the lack of reciprocal monophyly of O. vittatus since it is separated from the remaining Ommatotriton range by the Anatolian Diagonal (Fig. 1). This mountain range was formed at 30-25 Ma as a consequence of the Arabia-Eurasia collision (Jolivet & Faccenna, 2000) and acts as a barrier for a wide range of taxa (Kapli et al., 2013). Uplift of the Anatolian Diagonal likely isolated the stock that would become O. vittatus at ~25 Ma (Fig. 2b). Last Glacial Maximum conditions appear to have exacerbated this geographical isolation (Fig. 4).

The Arabia-Eurasia collision resulted in the uplift of the Anatolian Plateau (Hearn & Ni, 1994), which likely initiated speciation of what would become O. nesterovi and O. ophryticus at ~25 Ma (Fig. 2b). The two species were isolated during the Last Glacial Maximum (Fig. 4), implying that the current contact is of secondary, postglacial origin. Bilgin (2011) documented a north-south oriented suture zone, related to postglacial expansion

from refugia in western and eastern Anatolia for plants, insects and vertebrates. The two other pairs of parapatric newt species distributed along the southern Black Sea coast show evidence of hybridisation upon secondary postglacial contact with pronounced mtDNA introgression, from the western (Lissotriton vulgaris and Triturus ivanbureschi) to the eastern species (L. kosswigi and T. anatolicus; Nadachowska & Babik, 2009; Wielstra et al., 2013, in press), but there is no such mismatch between mtDNA and nuDNA for O. nesterovi and O. ophryticus. While we did not find evidence of (recent) introgression between the two species, to be conclusive a denser sampling, filling the 60 km gap between the closest O. nesterovi and O. ophryticus localities (localities 14 and 15, Fig. 1), is required.

4.2 Ancient and rapid radiation

The fossil based time calibration suggests the Ommatotriton species radiated ~ 25 Ma during a short time span. The mtDNA phylogeny suggests the three species form a polytomy. While our nuDNA species tree supported a ((O. ophryticus, O. vittatus), O. nesterovi) phylogeny, support decreased when adding mtDNA. We conclude that our data provide no convincing support of a particular branching order, and we consider the Ommatotriton phylogeny unresolved.

Conflicting results among markers can be expected for ancient and rapid radiations because few informative substitutions accumulate along internal branches (Philippe et al., 2011), little time is available for lineage sorting, resulting in conflicting gene genealogies (Pamilo & Nei, 1988) and uninformative substitutions accumulate along terminal branches, causing long-branch attraction (Felsenstein, 1978). Although comprehensive sampling of phylogenetic diversity can break up long branches (Bergsten, 2005), our sampling is comprehensive. However, we merely employed three unlinked genetic markers. Multispecies coalescence methods, as implemented in e.g. *BEAST, explicitly take incomplete lineage

sorting into account (Heled & Drummond, 2010) and benefit from sampling more unlinked markers (Maddison & Knowles, 2006). Hence, we suggest a phylogenomic approach is required to resolve the phylogeny of banded newts.

4.3 Deep intraspecific phylogeographical structure

A deep genetic differentiation within Ommatotriton species is reflected by four to five intraspecific mtDNA phylogeographical groups per species. Splits between O. nesterovi groups located southeast of the Sea of Marmara (A, B) and along the central southern Black Sea shore (C, D), and between O. vittatus groups from the Adana Basin and Israel (I, M) and Turkey east of the Amanus Mountains and Syria (J, K, L; Fig. 1, Fig. 2b), coincide with the Middle Miocene disruption, a sudden period of climate cooling between 14.0 and 13.5 Ma (Flower & Kennett, 1994). This period is linked to vicariance and genetic divergence in the Near East for skinks (Ablepharus; Skourtanioti et al., 2016), toads (Bufo; Garcia-Porta et al., 2012) and scorpions (Mesobuthus; Shi et al., 2013). The basal split between O. ophryticus groups located in central Turkey (E) and north-eastern Turkey, Georgia and on the western side of the Greater Caucasus (F, G, H) coincides with the uplift of the Armenian plateau during the Serravallian, caused by the Arabia-Eurasia collision (13-11 Ma; Gelati, 1975). This event was also inferred to have caused the split between the crested newts Triturus karelinii versusT. ivanbureschi and T. anatolicus (Wielstra et al., 2010).

We observe further subdivision into O. nesterovi groups (A and B, C and D), during the Late Miocene and Pliocene. This time period was associated with large-scale orogeny throughout the region (Oosterbroek & Arntzen, 1992; Bilgin, 2011). The different O. nesterovi groups are currently separated by protrusions of the Pontic Mountains, but the presence of a distinct haplotype from inside the range of group A, basal to the clade of groups A and B, shows the pattern is more complex. Divergence of the three groups distributed in

the eastern part of the O. ophryticus range (F, G, H) suggests the Quaternary climate oscillations (2.6 Ma - present) initiated increased population fragmentation.

For O. vittatus we observe two allopatrically distributed groups that, even though they are sister clades, occur in the Adana Basin (I) and Israel (M). The Adana Basin is isolated from the remainder of the Ommatotriton range by the Amanus Mountains lifted during the lower-middle Miocene (~23-11 Ma; Aksu et al., 2005). Desiccation of the Mediterranean Sea during the Messinian salinity crisis (~5.96-5.33 Ma) would have established a direct connection allowing colonization of the Adana Basin (Duggen et al., 2003). Subsequent expansion of the stock that became groups J, K and L would explain the disjunct distribution pattern observed today. Interpreting the phylogeographical pattern of groups J, K, and L is difficult based on the current sampling. Wider sampling in this presently hostile area would be required to further elucidate the historical biogeography of O. vittatus. Deep phylogenetic splits and complex phylogeographical patterns caused by the uplift of the Amanus Mountains and dispersal routes facilitated by the Messinian salinity crisis in southern Anatolia and the Levant were previously suggested for amphibians (Akin et al., 2010; Skourtanioti et al., 2016) and reptiles (Guicking et al., 2009; Kapli et al., 2013).

4.4 Multiple intraspecific glacial refugia

The species distribution models for O. vittatus suggest relatively little range contraction during the Last Glacial Maximum (Fig. 4), supported by the Bayesian skyline plot suggesting a relatively small population decrease during the Quaternary (Fig. B.12). The deep phylogeographical structure suggests multiple glacial refugia (i.e. “refugia-within-refugia”) were present within the current range of O. vittatus. Multiple refugia within the current range of O. vittatus have been found for reptiles (Kornilios et al., 2011; Stümpel et al., 2016), insects (Simonato et al., 2007) and plants (Médail & Diadema, 2009).

Compared to O. vittatus, a more pronounced habitat reduction at the Last Glacial Maximum was suggested by the species distribution models for O. ophryticus and, especially, O. nesterovi (Fig. 4). This is supported by the reduced population size indicated by the Bayesian skyline plots (Fig. B12). The deep genetic structure within the two northern species also survived the Last Glacial Maximum in multiple glacial refugia, located south of the Marmara Sea, and various locations along the southern Black Sea shore and in the Colchis (Fig. 1). The Marmara region acted as a glacial refugium for newts (Wielstra et al., 2010; Pabijan et al., 2015), multiple glacial refugia along the southern Black Sea coast were found for amphibians (Wielstra et al., 2010; Dufresnes et al., 2016), reptiles (Guicking et al., 2009) and mammals (Dubey et al., 2006, 2007; Bilgin et al., 2008) and multiple glacial refugia in the Colchis were found for plants, insects and snails (Tarkhnishvili et al., 2012), amphibians (Tarkhnishvili et al., 2000; Veith et al., 2003), lizards (Freitas et al., 2016), and mammals (Seddon et al., 2002; Tarkhnishvili et al., 2012).

CONCLUSIONS

The genus Ommatotriton represents a rapid radiation, initiated by orogeny associated with the Arabia-Eurasia collision during late Oligocene. Lack of gene flow supports the recognition of three species of banded newt, but relationships among these species remain unresolved. Each species is composed of distinct, geographically coherent mtDNA clades, with splits dated from the onset of the Quaternary to as far back in time as the Middle Miocene. Multiple glacial refugia per species are positioned along the coasts of the Black and Mediterranean Seas, suggesting a refugia-within-refugia scenario for the Near East, as has been previously inferred for the Iberian (Gómez & Lunt, 2007), Italian (Canestrelli et al., 2012) and Balkan peninsulas (Poulakakis et al., 2015). Ommatotriton is exemplary for co-distributed taxa and the overarching pattern emerging from cumulative phylogeographical studies underlines the

Near East as an important biodiversity hotspot, on par with hotspots revealed through comparative phylogeography for the better studied Mediterranean peninsulas (Riddle, 2016).

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Appendices

Additional information may be found in the online version of this article:

Appendix A. Supplementary Tables A.1-A.5

Appendix B. Supplementary Figures B.1-B.12

GenBank accession numbers are available in Table A.1. The raw and edited sequence data, in- and output for phylogenetic analyses and species distribution model analyses are available at the Dryad database:

Figure legends

Figure 1: Distribution of and sampling scheme for banded newts (genus Ommatotriton). Coloured dashed lines roughly delineate the three species' ranges and black dashed lines delineate mtDNA-based phylogeographical groups (A-M). Numbered dots (1-45) represent sampled localities and small dots additional localities used for species distribution modelling. Grey shading reflects elevation.

Figure 2: Mitochondrial DNA phylogenetic tree and dated tree for banded newts (genus Ommatotriton). Panel a) Bayesian consensus tree with Bayesian posterior probability value above and Maximum Likelihood bootstrap support value below branches. Asterisks indicate nodes supported with a posterior probability > 0.95 and a bootstrap support value > 80 and (values below the crown nodes of phylogeographical groups are not shown). Tips are haplotypes with codes corresponding to Table A.1 and localities in parentheses. Panel b) dated phylogeny with Bayesian posterior probability value left above branches (asterisk indicates posterior probability > 0.95) and node age on the left below with 95% HPD confidence interval in parentheses right of nodes. In a) and b) capital letters correspond to the

phylogeographical groups delineated in Fig. 1; the outgroup is not shown. Panel c) male banded newt species in breeding condition.

Figure 3: Haplotype networks and species tree based on nuclear markers for banded newts (genus Ommatotriton). In the haplotype networks for KIAA (a) and SACS (b) pie diameter represents the number of individuals per haplotype and bars the number of inferred substitutions. Panel c) *BEAST species tree with posterior values for all markers above, and only nuDNA markers below the branches. Colours indicate species as in Fig. 1.

Figure 4: Species distribution models for banded newts (genus Ommatotriton). Model projections for the three species are arranged from top to bottom, with projections on current (left) and MIROC-derived Last Glacial Maximum (right) climate layers. Predicted suitability is reflected by a colour scale running from deep blue (highly unsuitable) to deep red (highly suitable).

Figures

Figure 1

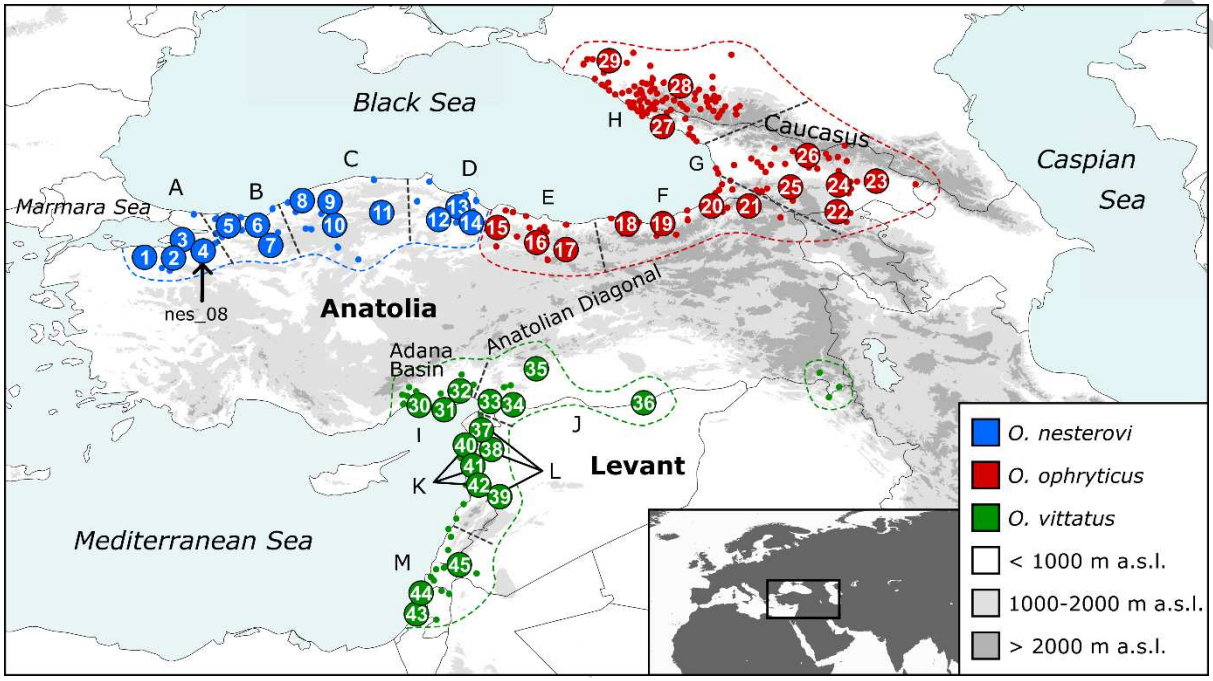


Figure 2

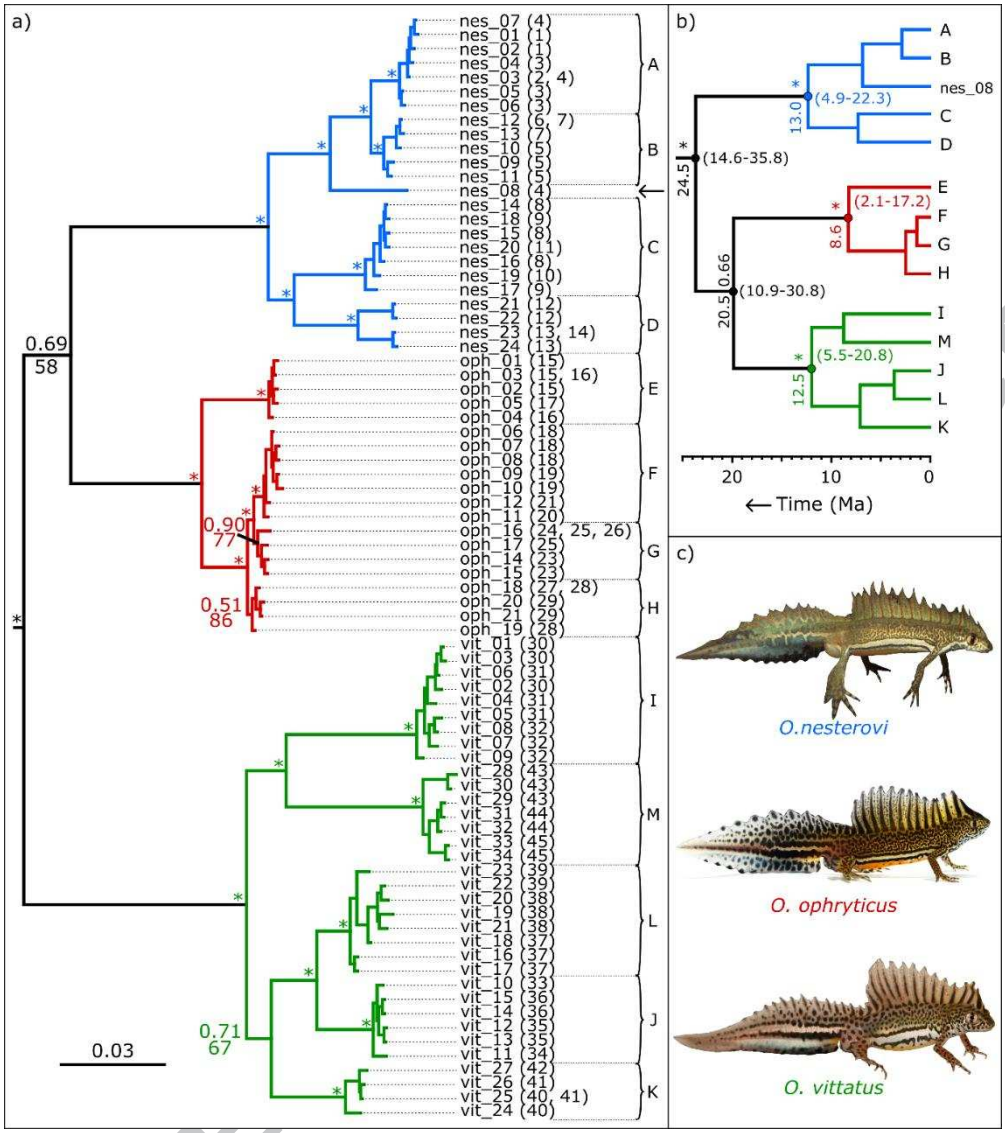


Figure 3

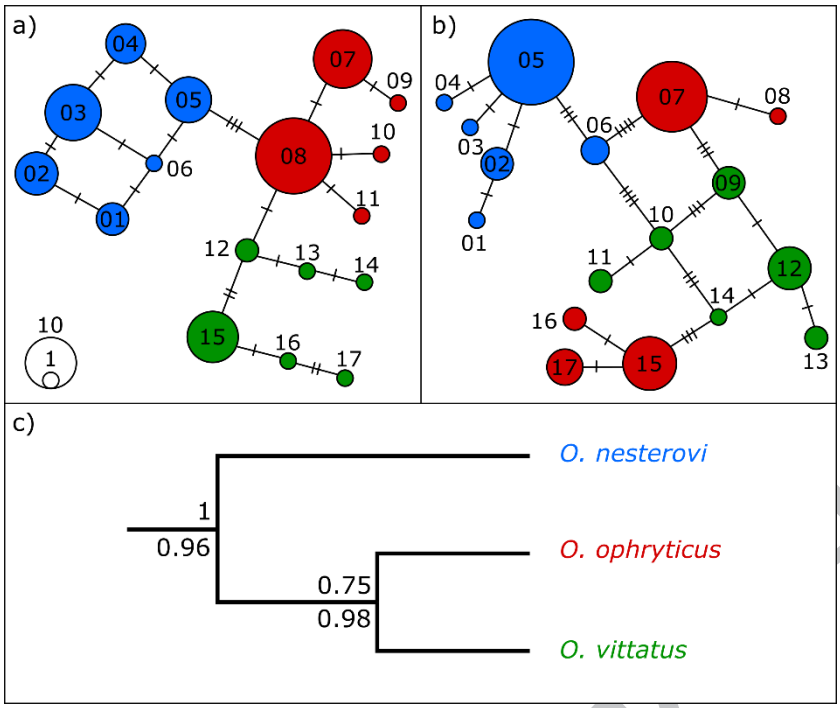
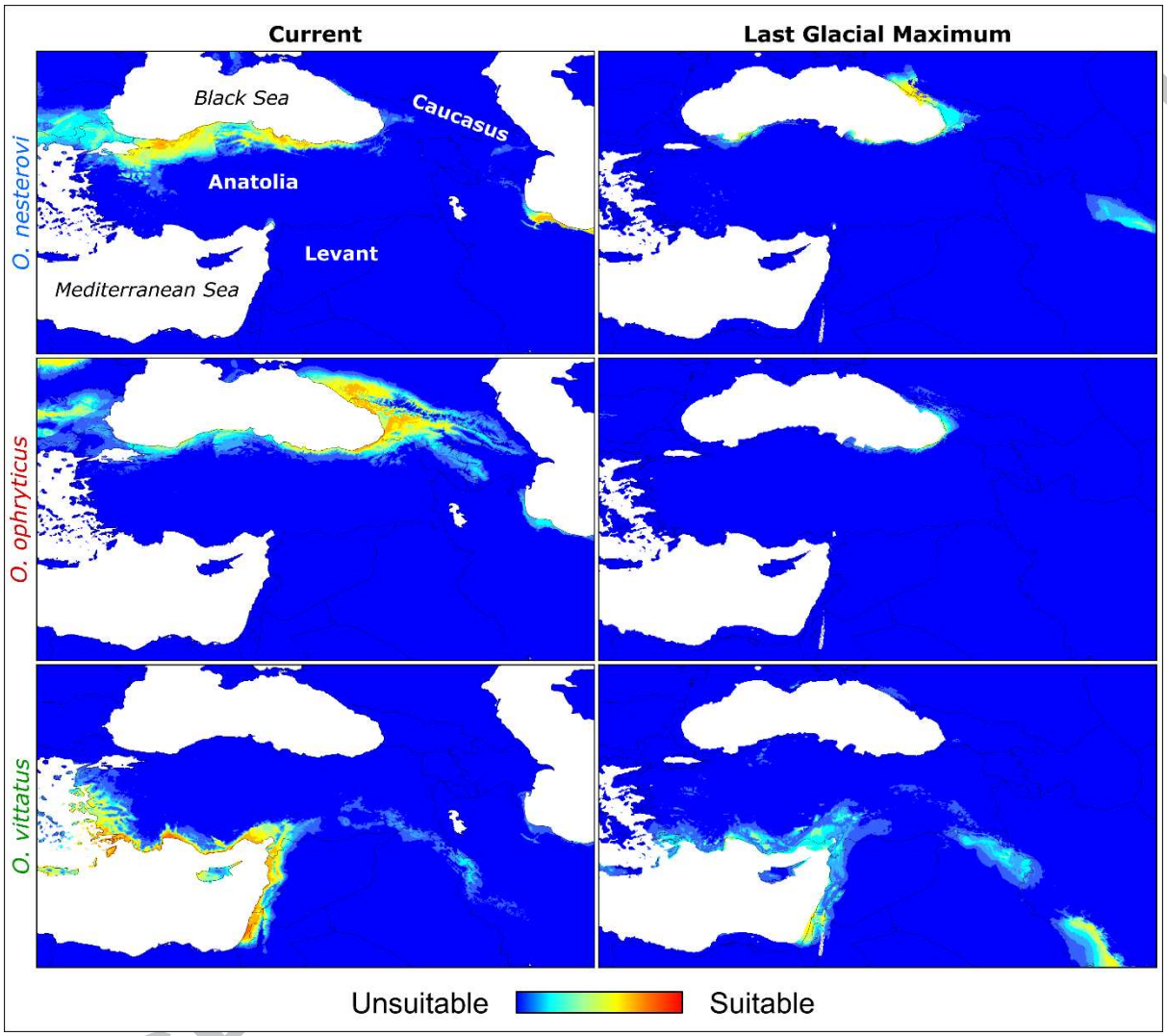
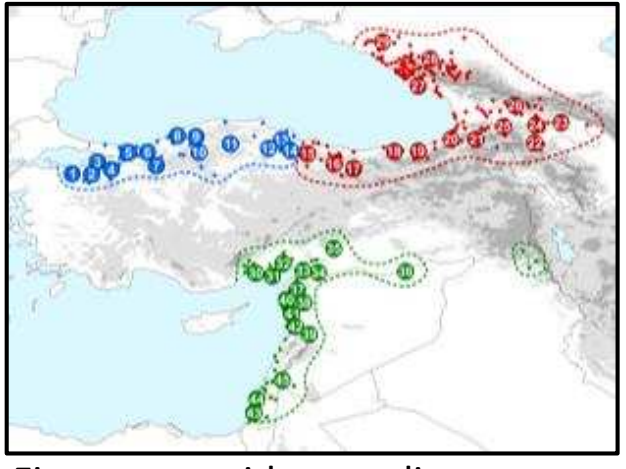


Figure 4

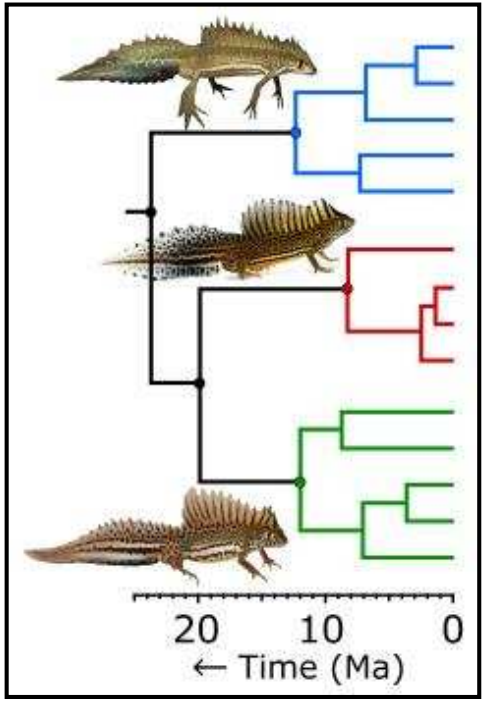


Genus *Ommatotriton*

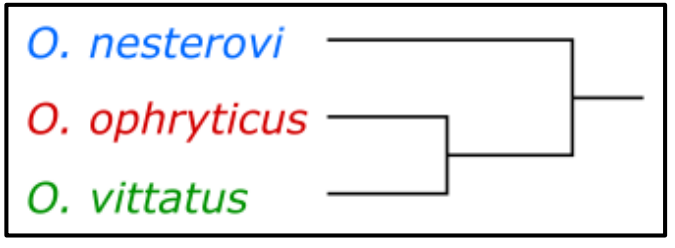


First range-wide sampling

MtDNA fossil calibrated tree



Fast and old species radiation



NuDNA species tree

Three genetically distinct species

Highlights

- The first range-wide phylogeography for the Near Eastern banded newt genus Ommatotriton.
- An old (~25 Ma) and rapid radiation of three genetically distinct species with unclear phylogenetic relationships.
- Intraspecific divergence is deep (crowns ~8-13 Ma) and no evidence for strong population bottlenecks was found.
- Long-term in situ evolution and survival in multiple glacial refugia underline the role of the Near East as biodiversity hotspot.